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PRINCIPAL INVESTIGATOR: Judith T. Zelikoff, Ph.D.

CONTRACTING ORGANIZATION: New York University School of Medicine
Tuxedo, New York 10987

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6. AUTHOR(S)
Judith T. Zelikoff, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
New York University School of Medicine
Tuxedo, New York 10987

E-MAIL:
judyz@env.med.nyu.edu

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13. ABSTRACT ***(Maximum 200 Words)***

Aquatic species are exposed to chemical contaminants that leach into the water from neighboring dump-sites or are directly discharged there. Heavily polluted water affects the health of aquatic life by, among other things, enhancing the incidence of infectious diseases. In light of the fact that: fish are directly-exposed in the water to toxic chemicals; the immune system is an extremely sensitive indicator for detecting the effects of toxic chemicals; and that the immune responses of fish are highly-conserved phylogenetically and, thus, structurally and functionally related to that of mammals, a study is proposed to test the hypotheses that immune functions of fish can serve as biological indicators for predicting toxicological hazards associated with polluted aquatic environments, as well as serve as an alternate model for mammals for predicting human health risks. The proposed studies employ a panel of well-established immune assays to evaluate the effects of known mammalian immunotoxicants and aquatic pollutants, alone and in combination, on the immune responses of Japanese medaka (*Oryzias latipes*). This study provides the opportunity to better understand the toxicological hazards and human health risks associated with exposure to militarily-relevant chemicals and meets the goals of the military which are to develop and validate new and more sensitive methods for assessing the toxic effects of chemicals in the ambient environment.

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FOREWORD

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Signature JZ 4/19/00

Table of Contents

FISH IMMUNE RESPONSES AS BIOMARKERS
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I. INTRODUCTION

A. Statement of Work - A study is proposed that will test the hypotheses that: fish can serve as an alternate species for mammals to predict potential human health risks; and, altered immune responses of fish can serve as a biological system for hazard assessment that can accurately predict toxicological risks associated with polluted aquatic environments.

This study will examine two inter-related parameters which are linked by their ability to provide information necessary for hazard assessment, as well as for providing a better understanding of human health risks associated with exposure to militarily-relevant chemicals. These aims include:

(1) To determine and compare the effects of mammalian immunotoxicants on the immune response of medaka and/or bluegill using a battery of well-established/validated immunological assays. These studies will be performed to determine how closely chemically-induced alterations in the immune responses of fish can predict effects produced in mammalian species. They will also provide information necessary for interspecies extrapolation and, ultimately, for predicting toxicological risk to both directly- and indirectly-exposed species. Effects of known mammalian immunotoxicants, as well as those lacking immunotoxic potential will be examined.

(2) To assess the toxicological hazards associated with contaminated water and/or sediment collected from a militarily-generated waste site. This Objective specifically addresses the question of whether immune assays developed/validated in this laboratory in medaka and/or bluegill (and used previously by our laboratory in field studies with smallmouth bass, brown/rainbow trout, and walleye) can be successfully employed to predict the toxicological hazards associated with militarily-generated waste sites. In addition, these investigations will further elucidate how markers of exposure (i.e., body burden) may relate to observed biological effects.

B. Background - Aquatic species are exposed to mixtures of chemical contaminants that are directly discharged into the water or leached from neighboring dump-sites. Heavily polluted water can affect directly-exposed species by enhancing disease incidence leading, potentially, to death of the host; adverse health consequences on humans that consume these affected organisms have also been observed. Because of this, fish systems are currently being used for biomonitoring studies to predict toxicological hazards associated with chemical exposure (1). In addition to their use for hazard assessment, much emphasis has recently been placed on the development of fish as alternate models for higher vertebrates in toxicological/immunotoxicological studies (2).

The sophistication and complexity of the immune system enables it to likely be the most sensitive and, therefore, most prominent body function to detect harmful effects from chemicals (3). Many of the same chemicals shown to alter immunoresponsiveness in mammals also act as immunotoxicants in fish, and in many cases bring about similar effects and act by like mechanisms. Some classes of chemicals shown to modulate immune responses of both species include: metals (i.e., cadmium, nickel, lead), pesticides/insecticides (malathion, pentachlorophenol), and polycyclic aromatic hydrocarbons [benzo(a)pyrene] (4).

Given the: sensitivity of the mammalian immune system to respond to low levels of environmental chemicals; morphological/functional/biochemical similarities between mammalian and fish immune defense mechanisms; and, the previously-observed ability of the proposed immune assays to successfully predict the immunotoxic effects of *in vivo* chemical exposure (5, 6, 7), it appears likely that results from these studies can be extrapolated to higher vertebrates for predicting toxicological risk and that immune responses of medaka/bluegill can be used successfully to predict the immunotoxic effects of militarily-contaminated aquatic environments.

II. BODY

Specific Aim 1

To determine the effects of well-studied mammalian immunotoxicants (and those lacking immunotoxic potential) in medaka/bluegill using a panel of well-established/validated immune assays.

Of the seven known mammalian immunotoxicants proposed in this aim for testing in medaka (*Oryzias latipes*) and/or bluegill (*Lepomis macrochirus*), three chemicals have either been completely evaluated or are currently being assessed; the three chemicals lacking immunotoxic activity in mammals will be examined in future studies.

A. Benzo(a)pyrene (BaP) Studies - Benzo(a)pyrene, a chemical in the class of polycyclic aromatic hydrocarbons (PAH), is a ubiquitous environmental contaminant with well-known immune and endocrine disrupting potential in a variety of mammalian species. While B(a) P and/or its metabolites has been shown in a number of previously-performed studies to affect T-lymphocytes (i.e., cell-mediated immunity) and antigen-presenting cells (i.e., macrophages), it appears that the major immunomodulating effects of B(a)P in mammalian species may be on humoral immunity and/or B-cell function. While the exact mechanism for B(a)P-induced alterations in B-cell function is unclear, it appears that macrophages (Mø) are involved. Moreover, it seems somewhat certain that it is the B(a)P metabolites (particularly B(a)P-7,8-diol and the diol epoxide) rather than the parent compound which is responsible for the observed immunosuppressive effects in mammals (8).

While the general toxicity of B(a)P on fish and CYP1A induction have been previously-described, very few studies have been performed examining the immunomodulating effects of B(a)P on piscine models (9). However, field studies of fish exposed to B(a)P-contaminated

environments have demonstrated suppressed T- and increased B-lymphocyte proliferative responses compared to fish recovered from an uncontaminated reference site (10). In other studies, *in vitro* and/or *in vivo* exposure to the PAH dimethylbenzanthracene suppressed natural killer (NK) cell and Mø activity, as well as total numbers of antibody-forming cells.

1. Intraperitoneal (IP) Exposure of Medaka- Because the effects of B(a)P on host immunocompetence were examined in many rodent studies using an IP exposure route (8), a similar route of exposure was employed for these fish studies.

(a) Host survival and enzyme induction/activity - In the first series of experiments, medaka were injected IP with either the corn oil control or with B(a)P at 2. 20. 200, 400, or 600 µg B(a)P/g body weight (BW) and effects upon fish survival determined. Even at the highest concentration, no effects were observed on host survival for up to 7d following injection (compared to the uninjected or vehicle-injected control).

To assure BaP uptake by fish, EROD activity and CYP1A protein were measured in the livers of exposed fish (11, 12, 13). As shown in Figures 1a and b, induction of cytochrome P4501A (as measured by Western blot analysis) and EROD activity (as measured by ELISA) were elevated (compared to control) in fish exposed to the two highest B(a)P concentrations; although effects upon EROD activity could not be evaluated statistically (due to small sample size), CYP1A induction was significantly elevated in exposed fish (compared to the vehicle-controls).

(b) Phagocyte-mediated superoxide production ($O_2 \cdot^-$) - While no effects were observed on extracellular $O_2 \cdot^-$ production (Figure 2a), phorbol myristate acetate (PMA)-stimulated intracellular $O_2 \cdot^-$ production (as measured by nitroblue tetrazolium reduction) was significantly reduced (compared to control) following IP injection of the highest tested BaP concentration (i.e. 200 µg/g BW); no effects upon $O_2 \cdot^-$ production were observed following IP injection of 2 µg BaP/g BW. Effects on intracellular $O_2 \cdot^-$ production correlated with changes in CYP1A protein levels (i.e., $R^2 = 0.982$) (Figures 2a and b).

(c) Antibody-forming cell (AFC) numbers - To begin to examine the effects of BaP on humoral immunity, medaka already-exposed to BaP were injected 48 hr later with sheep red blood cells (sRBC) and effects upon splenic AFC numbers determined by enumerating the number of plaques formed on a sRBC lawn. Injection of fish with 200, but not 2 µg BaP/g BW significantly reduced AFC numbers (Figure 3). In fish simultaneously exposed to sRBC and the CYP1A antagonist alpha-napthoflavone (14) PFC numbers were comparable to those measured in the vehicle-control. Results from these preliminary antagonist studies demonstrate the potential role of BaP metabolites in producing immune suppression; further studies using additional antagonists that act both upstream and downstream of CYP1A induction are planned. Like that observed for $O_2 \cdot^-$ production, dose-related effects of BaP on PFC numbers correlated with changes in CYP1A protein

levels (i.e., R^2 = 0.811). Similar suppressive effects on PFC numbers have also been observed in rodents exposed intraperitoneally to BaP (8).

(d) Lymphocyte Proliferation - Splenic lymphocytes recovered from vehicle control and BaP-injected medaka were stimulated *in vitro* with either the T-cell stimulant, Con A, or lipopolysaccharide (LPS) to stimulate T- and B-lymphocytes, respectively (Figures 4a and b). In both cases, lymphoproliferative responses were significantly depressed following exposure to all BaP concentrations tested; while BaP-induced effects upon B-cell proliferation appeared to be independent of dose, effects upon T-cell proliferation was dose-dependent. Of particular interest was that lymphoproliferative responses were altered by BaP at a dose 100-fold less than that required to induce CYP1A. Results of these studies suggest that lymphoproliferative responses may be a more sensitive indicator than CYP1A for predicting the adverse effects of BaP exposure.

(e) Thymic Cellularity - In studies performed to determine whether BaP can act in a similar manner to dioxin (i.e., polycyclic aromatic hydrocarbon vs. halogenated aromatic hydrocarbon) by causing thymic atrophy, thymocyte numbers were determined in exposed and vehicle-injected medaka two and seven days following a single IP injection of BaP at either 2, 200, or 600 µg/g BW (Figure 5a). Results of these studies demonstrated that exposure to BaP reduced thymic cellularity in medaka, but only after exposure to the highest dose (i.e., 600 µg/g BW). Reduction in thymic cellularity can be visualized histologically in Figure 5b.

Taken together, BaP-induced effects observed in medaka following IP exposure are similar to those previously-reported for rodents exposed via a similar route **(8).** Although effects upon B-cell proliferation in rodents are uncertain, both species have demonstrated reduced: lymphoid tissue cellularity; T-cell proliferation; macrophage activity; and, antibody-forming cell numbers in response to IP exposure to BaP. Further studies are planned evaluating the effects of BaP on host resistance against challenge with the infectious bacterial pathogen, *Yersinia ruckerii.*

2. Waterborne Exposure of Bluegill - Benzo(a)pyrene studies have been initiated with bluegill; effects were examined on immune endpoints identical to those studied in medaka (c.f. IIA). For these studies, bluegill were exposed for 48 hr to waterborne BaP at either 1, 0.5 or 0.1 ppm; no effects on host survival were observed at even the highest exposure concentration. In contrast to the dramatic suppressive effects observed in medaka, waterborne exposure to BaP at concentrations as high as 0.5 ppm had no effect upon lymphoproliferation (compared to control). However, extracellular $O_2^{\cdot -}$ production was affected similarly by BaP in both fish species. Waterborne exposure of bluegill to BaP at 0.5 ppm significantly reduced extracellular $O_2^{\cdot -}$ production by PMA-stimulated kidney phagocytes; no effects were observed at the lower waterborne BaP concentrations. Interestingly, intracellular $O_2^{\cdot -}$ production was increased in bluegill rather than decreased by BaP exposure (i.e., 0.1 ppm BaP). Plaque-forming cell numbers were depressed

in fish exposed to BaP at 0.5 ppm, but the effect appeared due to the vehicle rather than to the BaP itself. Future studies are being planned in which medaka will be exposed to waterborne BaP at concentrations equivalent to those injected in our previous studies.

B. Dexamethasone Studies - Because of the well-recognized potent immunosuppressive effects of the synthetic glucocorticoid dexamethasone (DEX) in mammalian species, medaka were exposed by IP injection to different DEX concentrations so as to validate the medaka model as an alternate species for mammals in immunotoxicity testing and for species extrapolation modeling. Dexamethasone, used in mammals for the treatment of allograft rejection, autoimmune reactions, and allergic and malignant disease, has been shown to inhibit: the production of interleukins; proliferation and function of T-lymphocytes; antibody production; cell-mediated immunity; and, host resistance against infection with a variety of protozoan parasites (15). However, in contrast to the plethora of information available for mammals, little is known concerning the effects of DEX on fish immunocompetence (16).

1. Host Survival - So as to define those sub-lethal doses of DEX that could be used to assess effects on immune function and host resistance, host survival LD_{50} studies were performed. For these studies, medaka were administered a single IP injection of either vehicle (i.e., phosphate buffered saline) or DEX at a concentration ranging between 0.5 and 5000 µg/fish; survival was monitored for up to six days post-injection. Injection of medaka with either 5000, 750 or 500 µg DEX/fish produced 100% lethality by twenty-four post-injection; administration of either 400 or 300 µg DEX/fish produced 53% and 13% mortality, respectively. Injection of DEX concentrations $\leq$ 200 µg/fish had no effect on medaka survival. Thus, all studies examining the effects of DEX on immune cell function employed concentrations $\leq$ 300 µg DEX/fish.

2. Immune Function Assays - To examine the effects of DEX on medaka immune responses, immune cells recovered from fish 48 hr following IP injection with either saline or DEX (i.e., 100, 200, or 300 µg/fish) were used to assess lymphocyte proliferation and $O_2^{\cdot -}$ production. Kidney cells recovered from DEX-exposed fish and stimulated *in vitro* with the T-cell mitogen concanavalin A (con A) demonstrated a 52% reduction in proliferative responses compared to those recovered from the vehicle control; effects appeared to be independent of exposure dose. Lipopolysaccharide (LPS)-stimulated B-cell proliferation was also suppressed by DEX exposure, but only at the highest injected concentration (i.e., 300 µg/fish). While these studies are currently being repeated, initial results suggest that T-cells and/or cell-mediated immunity in medaka is more sensitive to the suppressive effects of DEX than B-cells and/or humoral-mediated immune responses.

In contrast to effects observed on specific immunity, innate immune functions (as measured by phagocyte-mediated extracellular $O_2^{\cdot -}$ production) was enhanced 16% by DEX exposure (compared to the vehicle control); intracellular $O_2^{\cdot -}$ production was unaffected by DEX exposure. Studies are currently underway to assess effects of DEX on antibody-forming cell numbers and host resistance against the infectious bacterial pathogen, *Yersinia ruckerii.* While the aforementioned findings are based upon a small data set, results suggest that (under the conditions employed in this study) immune responses of medaka, like those of mammals, are sensitive to the immunomodulating effects of DEX.

All of the aforementioned *ex vivo* assays were carried out in three replicates. The mean values of the replicates were used in subsequent statistical analyses. Differences between the groups were analyzed using one-way ANOVA, followed, when appropriate, by Dunnett's test. Statistical significance was accepted at $p < 0.05$. No statistical analyses were carried out in pilot studies or in ongoing studies where the data set was too small for appropriate analyses.

C. Malathion Studies

Studies examining the effects of malathion on medaka immunocompetence were carried out in the laboratory of our collaborators at the U.S. Army Center for Environmental Health Research (Joseph Beaman at Geo Centers, Ft. Detrick, MD). Results from these studies have been recently published in 1999 in the *Journal of Toxicology and Environmental Health* (5) and is attached as an appendix to this Progress Report. Specific statistical analyses used for the malathion studies are described within the appended manuscript.

III. KEY RESEARCH ACCOMPLISHMENTS

- Intraperitoneal exposure of BaP alters the immune response of medaka in a similar manner and, possibly, by similar mechanisms for those reported for mammals.
- Benzo(a)pyrene-induced alterations in the immune response of medaka can be observed at concentrations well below those which produce overt toxicity, and in many cases, prior to induction of CYP1A.
- Immune assays most successful for predicting chemical-induced immunotoxicity in medaka have been identified.
- It has been determined that the immunotoxic potential of BaP in fish depends upon the route of chemical exposure and the particular fish species used.
- Cell-mediated immune functions appear to be more sensitive to the immunomodulating effects of DEX than humoral-mediated immunity in medaka.

- Similar to those effects observed in rodents, exposure of medaka to malathion at environmentally-relevant concentrations suppresses both non-specific and specific immune responses.

IV. REPORTABLE OUTCOMES

A. Manuscripts/Abstracts/Presentations:

1. Manuscripts:

Zelikoff, J.T., Raymond, A., Carlson, E., Li, Y., Beaman, J.R., and Anderson, M. 2000. Biomarkers of immunotoxicity in fish: From the lab to the ocean. *Toxicol. Lett.* 112-113-325-331.

Zelikoff, J.T., Carlson, E., Li, Y., Raymond, A., and Beaman, J.R. 2000. Immune system biomarkers in fish for predicting the effects of environmental pollution. *Proceedings of The Fourth Princess Chulabhorn International Science Congress: Chemicals in the 21st Century.* pp. 13-24.

2. Abstracts:

Carlson, E.A., and Zelikoff, J.T. 2000. Acute *in vivo* exposure to the mammalian immunotoxicant benzo(a)pyrene alters immune responses of fish. *Toxicol. Sci* (supplement) 54:200.

Carlson, E.A., and Zelikoff, J.T. 1999. The teleost immune system: A sensitive biomarker of polycyclic aromatic hydrocarbon (PAH) toxicity. *Soc. Environ. Toxicol. Chemistry.* 20:265.

Raymond, A.R., Li, Y., Carlson, E., and Zelikoff, J.T. 1999. Use of immunotoxicity endpoints in fish for predicting aquatic pollution. *Proceedings of Hudson/Delaware Regional Chapter of SETAC.* 56. (**Awarded 2nd Place for Best Student Presentation).**

Carlson, E., and Zelikoff, J.T. 1999. Polycyclic aromatic hydrocarbon immunotoxicity in fish. *Proceedings of Hudson/Delaware Regional Chapter of SETAC.* 56.

Zelikoff, J.T., Raymond, A., Carlson, E., Li, Y., Beaman, J., and Anderson, M . 1999. Immunotoxicity biomarkers in fish: Development, validation, and application to field settings. *Proceedings of the Aquatic Toxicity Workshop.* 12.

3. Presentations

The Fourth Princess Chulabhorn Science Congress- Immune system biomarkers for predicting the effects of environmental pollution. Bangkok, Thailand. November 29, 1999.

Aquatic Toxicity Workshop **(SETAC, Canada)** - Immunotoxicity biomarkers in fish: Development, validation, and application to field settings. Edmonton, Canada. October 5, 1999.

Eurotox '99- Biomarkers of immunotoxicity in fish: From the lab to the ocean. Oslo, Norway. June 29, 1999.

The American College, India Academy of Science. Impact of Aquatic Pollution on Animal Health. Madurai, India. February 7 1999.

The American College - Immunotoxicity in fish. Madurai, India. February 9,1999.

B. Employment/Research Opportunities Supported by this Award -

Andrea Raymond - Master's Degree supported by this award and an Army Augmentation Award. Student currently pursuing a Ph.D. at Temple University Medical School in the Department of Microbiology/Immunology.

Collette Prophetti - Pursuant of a Master's degree in this work (studies in progress).

Jessica Duffy - Pursuant of a Master's degree in this work (studies in progress).

Migali Jorge - Pursuant of a Master's degree in this work (studies in progress).

V. CONCLUSIONS

The aforementioned studies have been carried out so as to determine how closely chemically-induced alterations in the immune responses of medaka/bluegill resemble those produced in mammalian species. Results of these comparative investigations have provided baseline information needed for interspecies extrapolation and for determining how useful assays which measure altered immune responses of fish are for predicting immunotoxicity in higher vertebrates (including humans).

A battery of immune assays previously established by the National Toxicology Program for identifying chemically-induced immunosuppression in rodents (and developed/validated in this laboratory for use in medaka/bluegill) were used to determine immunotoxicity in fish. The selected immune functional endpoints have a moderate to high predictive value for immunotoxicity (immunosuppression) in rodents and assess immune parameters important for maintaining humoral, cell-mediated, and/or non-specific aspects of host defense.

The military is concerned with evaluating environmental effects and human health risks associated with militarily-generated/released material that is subject to remediation through installation-restoration operations. To develop these required military criteria research is needed to develop and validate new, quicker, more economical methods (i.e., biomarkers/bioindicators) for assessing the fate, effects, and toxicity of chemicals in air, water, and terrestrial ecosystems. Thus, these studies meet the research needs of the military by applying well-established immune assays to assess the toxicological impact of polluting chemicals.

The use of immune biomarkers to predict the biological effects of pollutant exposure also provides the opportunity to rapidly evaluate potential far-reaching health effects associated with pollutant exposure both to those directly-exposed aquatic species and, potentially, to humans. These types of biomarker studies offer distinct advantages over endpoints which simply assess the occurrence of toxic pollutants and fail to determine potential health risks. Thus, this study clearly

meets the military's goals of evaluating environmental effects and human health risks (based on toxicological hazard) associated with militarily-generated/released materials.

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Hepatic CYP1A Protein Induction and EROD Activity in Fish Following In Vivo B(a)P Exposure

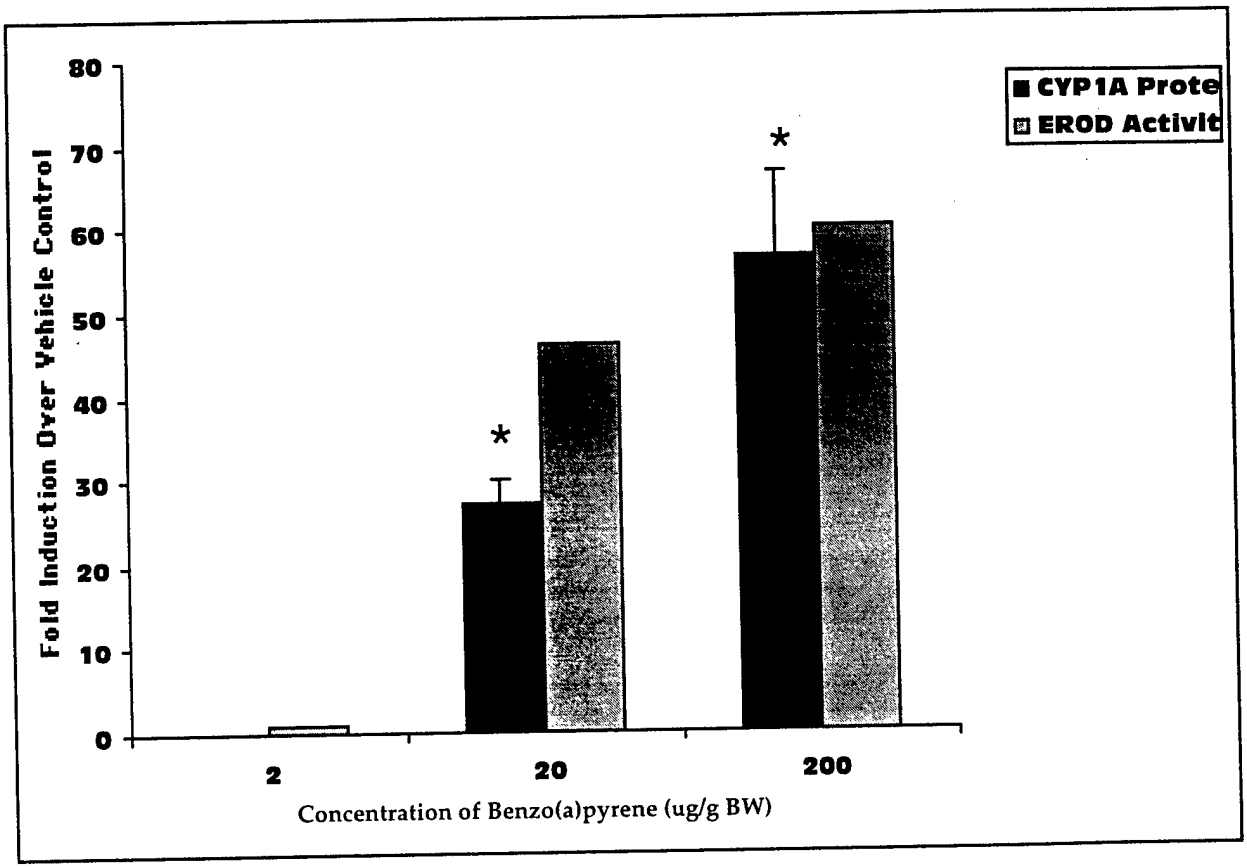
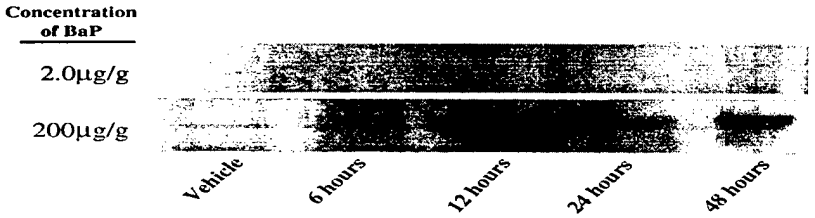


Figure 1a.

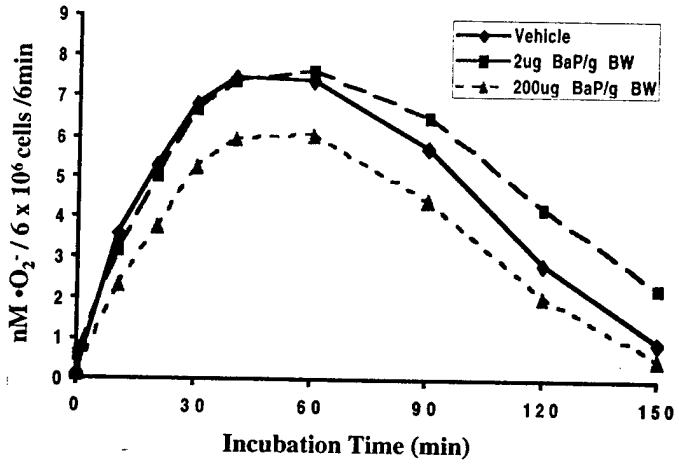
Hepatic CYP1A Induction Following *in vivo* Benzo(a)pyrene Exposure [a]



[a] CYP1A protein was measured by Western Blotting

Figure 1b.

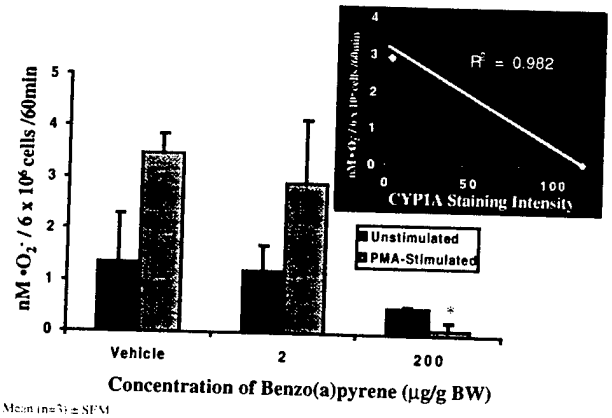
PMA- Stimulated Phagocyte Extracellular $\bullet O_2^-$ Production in Medaka Following IP Injection of Benzo(a)pyrene



* n = 1 for this Figure

Figure 2a.

PMA-Stimulated Intracellular $\bullet O_2^-$ Production was Reduced by 200µg BaP/g BW Exposure [a]



[a] Mean (n=5) ± SEM
* Significantly different from vehicle control (p<0.05)
Inset represents correlation between changes in immune parameters and CYP1A protein

Figure 2b.

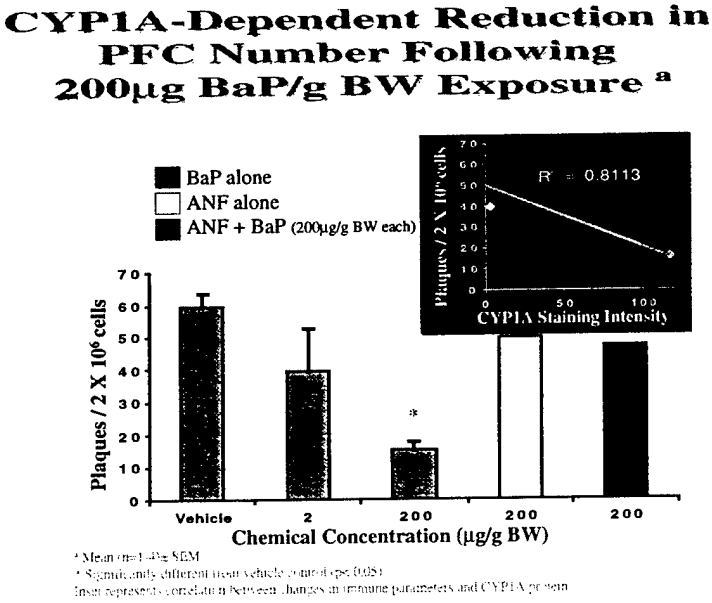
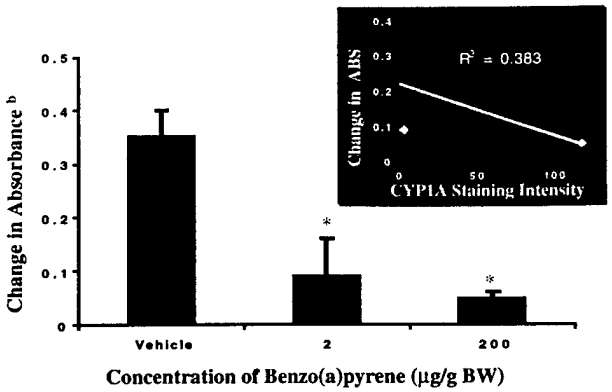

CYP1A-Dependent Reduction in PFC Number Following 200µg BaP/g BW Exposure a
BaP alone
ANF alone
ANF + BaP (200µg/g BW each)
Plaques / 2 X 10⁶ cells
Chemical Concentration (µg/g BW)
Vehicle
2
200
200
200
R² = 0.8113
CYP1A Staining Intensity
a Mean ± 1 SEM
* Significantly different from vehicle control (p≤ 0.05)
Inset represents correlation between changes in immune parameters and CYP1A protein

Figure 3.

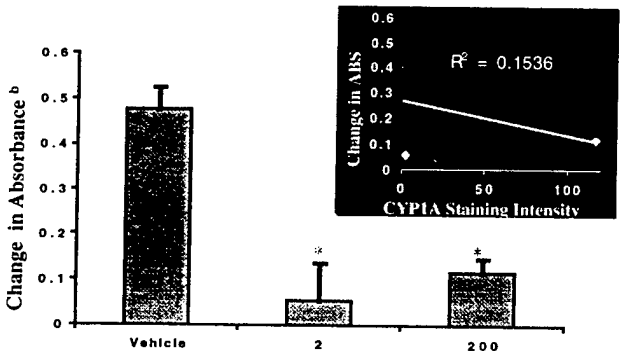
IP Injection of Benzo(a)pyrene Suppressed Con A-Stimulated T-Lymphocyte Proliferation [a]



[a] Mean (n=3) ± SEM
[b] (Absorbance with Con A) - (Absorbance without Con A) following 4d incubation
* Significantly different from vehicle control ($p \leq 0.05$)
Inset represents correlation between changes in immune parameters and CYP1A protein

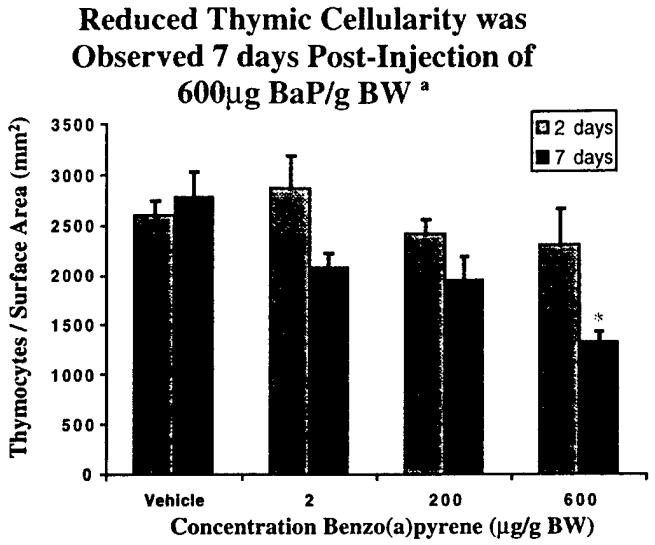
Figure 4a.

IP Injection of Benzo(a)pyrene Suppressed LPS-Stimulated B-Lymphocyte Proliferation [a]



[a] Mean (n=3) ± SEM
[b] (Absorbance with LPS) - (Absorbance without LPS) following 4d incubation
* Significantly different from vehicle control ($p \leq 0.05$)
Inset represents correlation between changes in immune parameters and CYP1A protein

Figure 4b.



[a] Mean (n = 3-8) ± SEM
* Significantly different from time-matched vehicle control ($p<0.05$)

Figure 5a.

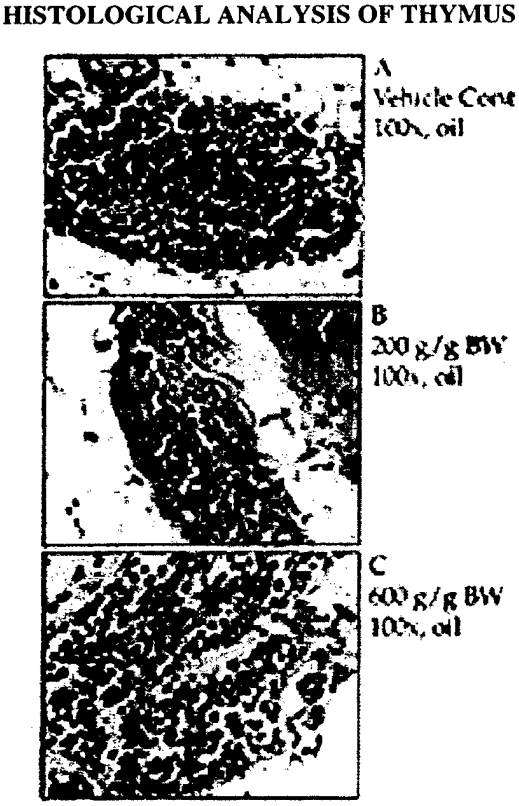


Figure 5b.

MAMMALIAN IMMUNOASSAYS FOR PREDICTING THE TOXICITY OF MALATHION IN A LABORATORY FISH MODEL

J. R. Beaman

Geo Centers Inc., Ft. Detrick, Frederick, Maryland, USA

R. Finch, H. Gardner, F. Hoffmann, A. Rosencrance

U.S. Army Center for Environmental Health Research, Ft. Detrick, Frederick, Maryland, USA

J. T. Zelikoff

New York University School of Medicine, Department of Environmental Medicine, Tuxedo, New York, USA

*This study describes the use of a panel of immune assays, originally developed by the National Toxicology Program for assessing xenobiotic-induced immunotoxicity in mice, to quantify the effects of sublethal malathion exposure on the immune responses of fish. For this study, Japanese medaka (*Oryzias latipes*) were exposed subchronically to the organophosphate pesticide malathion in a series of two experiments. In the first set of studies, fish were exposed for 7 or 14 d to untreated well water (i.e., controls) or to waterborne malathion at 0.2 or 0.8 mg/L. Following exposure, fish from each group were sacrificed and their kidneys (primary organ of leukopoiesis in fish and equivalent to mammalian bone marrow) were used to provide cells for assessing any malathion-induced effects upon nonspecific and acquired immune defense mechanisms. Effects upon humoral-mediated immunity were determined by enumerating antibody plaque-forming cell (PFC) numbers from a subset of fish exposed to malathion for 14 d and then injected intraperitoneally (ip) with sheep erythrocytes (sRBC). Results of these studies demonstrated that while malathion exposure had no significant effect upon hematocrit/leukocrit values or upon mitogen-stimulated T-cell lymphoproliferation, PFC numbers in the kidney of exposed fish were significantly reduced (compared to control fish) in a dose-dependent manner. In addition, total recoverable kidney cell numbers*

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The views, opinions, and/or findings contained in this report are those of the author(s) and should not be construed as official Department of the Army position, policy, or decision, unless so designated by other official documentation. Research was conducted in compliance with the Animal Welfare Act, and other federal statutes and regulations relating to animals and experiments involving animals and address to principles stated in the "Guide for the Care and Use of Laboratory Animals," NIH publication 86-23 (1985).

Address all correspondence to Dr. Judith T. Zelikoff, New York University School of Medicine, Nelson Institute of Environmental Medicine, 57 Old Forge Road, Tuxedo, NY 10987. E-mail: judyz@charlotte.med.nyu.edu

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and viability, as well as superoxide anion production by kidney phagocytes, were reduced slightly (compared to control values) in fish exposed for 14 d to the highest malathion concentration tested. In the second set of experiments, medaka exposed for up to 21 d to either 0.1 or 0.3 mg malathion/L were challenged ip with an LD50 dose of the bacterial fish pathogen Yersinia ruckeri. *Results from these infectivity studies demonstrated that exposure to either malathion concentration for 14 or 21 d reduced host resistance against* Yersinia *infection. Taken together, these findings demonstrate the applicability of mammalian immune assays for predicting malathion-induced immunosuppression in a teleost fish, as well as the potential utility of a small laboratory fish to serve as an alternate model for mammals in immunotoxicological studies.*

Historically, immunocompetence has been monitored using a tiered approach that is based upon several parameters including immunopathology, immune function, and host resistance. As described by Luster et al. (1988), well-characterized immune assays validated in rodents for their sensitivity and reproducibility in assessing xenobiotic-induced immunotoxicity are currently available. The National Toxicology Program (NTP) recommends this panel of assays for assessment of the responsiveness and functionality of immunocompetent cells, the effective cooperative interactions of these cells to produce cell- or humoral-mediated immunity, and the general immunocompetence of the host when challenged by infectious agents or tumor cells. Of late, many of these same immune assays have been used to assess chemical-induced immunotoxicity in animal species other than mammals (Weeks et al., 1992; Zelikoff, 1994a, 1994b, 1998; DeGuise et al., 1995; Luebke et al., 1997). The current studies are aimed at defining the usefulness of these assays for predicting the immunotoxic effects of malathion in a laboratory fish model.

Malathion [diethyl(dimethoxyphosphinothioylthio)succinate] is a nonsystemic, wide-spectrum, organophosphate insecticide that is used worldwide to control sucking and chewing insects on fruits and vegetables, disease vectors such as mosquitoes and flies, and ectoparasites of various animal species (Barnett & Rodgers, 1994). While the most well-studied effects of malathion are associated with its ability to inhibit acetylcholinesterase (AChE) activity, likely through binding of its malaxon metabolite to AChE (Shao-nan & De-fang, 1996), and to disrupt nerve impulse transmission (Richmonds & Dutta, 1992), malathion has also been shown in a number of in vivo and in vitro studies to modify mammalian host defense mechanisms (Zhou, 1989; Rodgers & Ellefson, 1990; Flipo et al., 1992; Rodgers et al., 1996; Rodgers, 1996; Rodgers et al., 1986). For example, increased respiratory burst capacity of recovered macrophage and enhanced mast-cell degranulation were observed in mice exposed orally for 14 d to malathion concentrations between 0.1 and 1 mg/kg/d (Rodgers & Xiong, 1997). Moreover, acute and subacute exposure to what appeared to be noncholinergic doses of malathion has been shown to significantly elevate the proliferative responses of murine splenocytes to exogenously added mitogens (Rodgers et al., 1996). In vivo exposure to malathion has also been shown

to elevate phorbol myristate acetate (PMA)-stimulated hydrogen peroxide (H_2O_2) production. Results of in vitro studies on immune function are less clear. While in vitro exposure of PMA-stimulated murine peritoneal cells to malathion reduced H_2O_2 production (Rodgers & Ellefson, 1990), exposure to malathion doses ranging between 100 and 500 mg/ml either suppressed or had no effect upon the proliferative responses of murine splenocytes or upon human peripheral blood mononuclear cells, respectively.

Even though significant amounts of malathion enter into the aquatic environment by direct application, spray drift, aerial spraying, atmospheric washing, erosion, and runoff from agricultural sites (Rodgers, 1996), and fish appear even more sensitive than mammals to the toxic effects of malathion (Johnson & Wallace, 1987; Shao-nan & De-fang, 1996), relatively little information is available concerning its biological impact upon inhabiting aquatic species (Mukhopadhyay & Dehadrai, 1982; Areechon & Plumb, 1990; Alam & Maughan, 1992).

As in their mammalian counterparts, exposure of teleost fish to sublethal concentrations of malathion appears to alter host immunoresponsiveness. While the amount of available information is extremely limited, studies have demonstrated that channel catfish (*Ictalurus punctatus*) exposed for 30 d to either 0.5 or 1.7 mg malathion/L exhibited depressed agglutination antibody titers against the infectious bacteria *Edwardsiella ictaluri* (Plumb & Areechon, 1990a). Studies by these same investigators (Plumb & Areechon, 1990b) also demonstrated that acute exposure of channel catfish to 4.5 mg malathion/L significantly decreased (compared to controls) circulating white blood cell (WBC) numbers in fish examined 48, 72, and 96 h post-exposure. These hematological findings were supported by the findings of Dutta et al. (1992) which demonstrated that waterborne exposure of Indian catfish (*Heteropneustes fossilis*) to 1.2 mg malathion/L (1/10 the 96-h LD50 value) produced leukopenia in fish exposed for 72 h; in contrast, exposure to the same malathion concentration for only 24 h increased circulating WBC numbers. Elevated total and differential WBC counts have also been observed in Indian catfish exposed chronically (i.e., 40 d) to a concentration of malathion at approximately half that used in the previous study (i.e., 0.5 mg malathion/L) (Mishra & Srivastava, 1983). Discrepancies between the aforementioned studies could be due to differences in temporality or exposure dose, since high (i.e., cholinergic) doses of malathion have been shown to suppress rodent immune responses while low concentrations enhance host immunocompetence (Barnett & Rodgers, 1994).

The aim of this study was to determine whether particular immune assays originally developed/validated in mice for demonstrating chemical-induced immunotoxicity could be used successfully in a small laboratory fish to illustrate the immunomodulating potential of a widely used organophosphorus pesticide. Results demonstrated that not only could the same NTP-validated immune assays be used successfully in Japanese

medaka to demonstrate the immunosuppressive effects of malathion, but that those assays proven most sensitive and predictive of xenobiotic-induced immunotoxicity in mammalian systems (Luster et al., 1988, 1994; Karol, 1998) were also the most responsive to malathion-induced effects in fish.

MATERIALS AND METHODS

Fish

Eight- to 9-mo-old Japanese medaka (*Oryzias latipes*) maintained at the U.S. Army Center for Environmental Health Research (USACEHR) were used for these studies. Fish were held in conditioned well water (at 25°C ± 1) in a 16:8 light:dark photoperiod under flow-through (100 ml/min) conditions in 42-L (10-gal) aquaria. Medaka, maintained at a stocking density of 60 fish/tank, were fed a diet of brine shrimp once daily and Aquatox flake food twice daily. Fish used for the malathion experiments were weighed and then randomly transferred from their holding facility at USACEHR to a nearby exposure aquaria located in a mobile biomonitoring laboratory. To provide ample time for recovery from any handling/transporting stress, fish were allowed to acclimate for 7 d before being used in any experiments. During this acclimatization period medaka were checked three times daily and any moribund fish were removed. Water quality was assured by evaluating temperature, pH, and dissolved oxygen daily; water hardness and free ammonia were monitored colorimetrically using commercially available kits (LaMotte, Chestertown, MD) at the initiation and termination of toxicant exposure. Levels of malathion in uncontaminated well water were measured by gas chromatography and found to be below detectable limits (i.e., 0.2 mg/L).

Chemicals, Reagents, and Bacterial Pathogen

Brain–heart infusion agar (BHIA) and tryptic soy agar and broth (TSA and TSB, respectively) used to culture/maintain bacteria were purchased from Baxter Scientific (Columbia, MD). Malathion (determined by supplier to be 99.8% pure) was purchased from Chemservice (Philadelphia, PA). Sheep erythrocytes (40% in Alsever's solution) and lyophilized guinea pig sera (used as the complement source for the plaque-forming cells assays) were purchased from Bio-Whittaker, Inc. (Walkersville, MD). All remaining buffers, chemicals, and reagents were purchased from Sigma (St. Louis, MO).

Yersinia ruckeri (strain 11.40), an opportunistic gram-negative bacteria that causes enteric redmouth disease in salmonids and other teleost fish species (Furones et al., 1993), was kindly provided by Dr. Jeff Teska (National Fish Health Laboratory, Leetown, WV). Bacterial stocks were grown in four 250-ml Erlenmeyer flasks (each containing ~200 ml TSB)

maintained in a 25°C shaking water bath. After 48 h, flasks were centrifuged for 10 min at 2000 rpm and the pelleted bacteria (in log-phase growth) were resuspended in 1 ml TSB. Bacteria were either used immediately for the initial LD50 range-finding studies or aliquoted (1 ml) and frozen (–70°C) until needed.

Malathion Preparation and Analytical Analyses

Malathion stock (120 mg/L) was prepared daily in 18 MΩ (deionized) water. The prepared solution was then pumped into a proportional diluter system using a standard digital drive peristaltic pump fitted with an Easy-Load pump head and Master-Flex platinum-cured silicone tubing (Cole-Palmer, IL). The flow-through exposure system utilizing the Envirotox flow-through bioassay diluter (Specialized Environmental Equipment, Inc., Easley, SC) consisted of a proportional diluter system where flow was controlled by selenoid valves that operated in conjunction with a timer set for 3-min cycles.

Water samples were recovered from each tank throughout the entire exposure period and the levels of malathion analyzed by gas chromatography. Ten-milliliter aliquots of malathion-contaminated water samples and standards were extracted with 2 ml of hexane-containing diazinon (200 mg/L) used as an internal standard (IS). Aliquoted samples, vortexed at high speed for 2 min, were then allowed to stand for an additional 5 min to permit separation of the organic layers. The hexane (top) layer was analyzed by gas chromatography using a Hewlett-Packard model 6890A gas chromatograph equipped with an electron capture detector (ECD), flame ionization unit, and a model 763A automatic sampler (Hewlett Packard Corp., Avondale, PA). Samples were processed at a flow rate of 60 ml/min under a column head pressure of 15 psi. Peak areas for malathion (and for the IS diazinon) were converted to a ratio, and the ratios for all working standards were plotted against the concentration of malathion in the working standards. Areas under the respective peaks were calculated using a computer regression fit model on the Hewlett-Packard Chemstation software program.

Experimental Design and Exposure Regime

Fish were exposed to malathion in 12 21-L (5-gal) glass aquaria containing filtered (reverse osmosis), ultraviolet-sterilized well-water from a local aquifer. Two sets of experiments were carried out to determine the effects of subchronic malathion exposure on the immune responses of Japanese medaka. In the first series of experiments, 720 medaka were randomized (7 d prior to exposure) into 12 aquaria (60 fish/aquaria; 4 aquaria/treatment group/exposure time point). Medaka, exposed for either 7 or 14 d to uncontaminated well water (untreated controls) or to malathion at 0.2 or 0.8 mg/L, were either sacrificed immediately after exposure and the kidneys removed to supply cells for assessing malathion-induced effects upon non-

specific and cell-mediated immunity, or were inoculated ip with 20 µl of sheep erythrocytes (sRBC) to assess effects upon humoral immune functions. Following injection with sRBC, fish were placed into clean well water for an additional 11 d and then sacrificed to enumerate kidney PFC numbers.

Nominal malathion concentrations of 0.2 and 0.8 mg/L were selected for use throughout the first set of immune function studies and are herein referred to as the "low" and "high" exposure doses, respectively. These malathion concentrations were selected based upon results from a previously-performed pilot study to assess the range of effects and the 96 h LC50 value. The LC50 value and the no-observable-effects level (NOEL) were calculated using d 0 gas chromatography-analyzed samples and estimated 96-h sample concentrations; estimation was based upon a malathion degradation rate of 50% in well water under static conditions (A. Rosencrance, personal communication).

In a second set of experiments designed to examine the effects of malathion upon host resistance, 480 medaka were randomized into 12 aquaria (40 fish/aquaria; 4 aquaria/treatment group/exposure time point) and, after acclimatization for 7 d, exposed to either uncontaminated well water or to well water containing 0.1 or 0.3 mg malathion/L. Malathion doses below those that produced mortality in the first set of experiments (i.e., 0.8 mg malathion/L) were selected for use in these studies. Following exposure to either 0.1 or 0.3 mg malathion/L or uncontaminated well water for 7, 14, or 21 d, 40 medaka/treatment group (10 fish/aquaria) were injected ip with a single dose of *Y. ruckeri* approximating the calculated LD50 value (i.e., 1.7×10^7 colony-forming units, CFU) in normal healthy fish; medaka injected ip with Hanks balanced salt solution (HBSS) served as the vehicle control. Survival of bacterially-infected medaka, maintained in 3.1-L animal jars (10 fish/jar; 4 jars/treatment/exposure duration) containing uncontaminated aerated water, was monitored over a 96-h time period. The 96-h mortality incidence was determined by assessing the number of fish surviving in a given exposure treatment group per total number of exposed fish. Medaka that succumbed within the first 3 h of injection were not included in the calculations, as death was considered to be related to injection rather than pathogen induced.

In Vivo Studies

Cell Isolation Kidney cells needed for the first series of experiments were isolated as described previously by Zelikoff et al. (1996). Briefly, organs disrupted by homogenization with a glass/glass tissue grinder were passed through a syringe barrel containing ~2 cm loosely packed sterile glass wool to remove red blood cells and debris. Cell counts and viability were performed by hemocytometer counting and trypan blue exclusion, respectively. Cells numbers were adjusted in either HBSS or L-15 medium to either: 4×10^6/ml for measurement of extracellular $O_2^{\bullet -}$ production; 5×10^6/

ml to assess mitogen-stimulated T-lymphocyte proliferation; 6×10^6/ml to assess H_2O_2 production and intracellular $O_2^{\bullet -}$ production; or 4×10^7/ml to enumerate antibody PFC numbers.

Immune Function Assays Functional assays performed in the first set of experiments, including intra- and extracellular superoxide ($O_2^{\bullet -}$) production, hydrogen peroxide (H_2O_2) formation, lymphoproliferation, and plaque-forming cell (PFC) response, were performed as described previously (Zelikoff, 1994a; Zelikoff et al., 1996; Twerdok et al., 1996). Those assay protocols not fully described elsewhere are briefly described below.

Mitogen-stimulated lymphoproliferation was performed by adding 5×10^5 kidney cells in supplemented L-15 medium (medium plus 1% sodium pyruvate, 1% nonessential amino acid solution, 0.01% mercaptoethanol, and 0.2% gentamicin) to individual wells of a 96-well flat-bottom microtiter plate. To stimulate T-cell proliferation, concanavalin A (Con A; 200 µg/ml) was added to the appropriate wells and the plates incubated for 72 h in a humidified 30°C chamber. Following incubation, 20 µl of 3-(4,5 dimethylthizol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT; 5 mg/ml) was added to each well and the plates were incubated for an additional 4 h. Fifty microliters of a 10% sodium dodoecyl sulfate (SDS)/0.01 *N* HCl solution (in double-distilled water) was then added to each well and the plates incubated for 24 h. Proliferation was determined colorimetrically using a spectrophotometer equipped with a 595-nm filter. After subtracting absorbance values for the reagent blank from each treatment, the proliferation index (PI) was determined by subtraction of the OD values from wells containing cells without stimulation from those measured in wells containing mitogen.

Antigen used to quantitate antibody PFC numbers was prepared by adding 2 ml of 40% sRBC (in Alsever's solution) to 6 ml ice-cold phosphate-buffered saline (PBS; pH = 7.2). After 6 washes, PBS was added in a sufficient quantity to dilute the concentration of sRBC to 10%. Medaka were then sensitized by ip injection with 20 µl of the washed antigen. After allowing 11 d for a primary antibody response to develop, kidneys from control and malathion-exposed fish were harvested and PFC numbers were assessed using a modification of the Jerne plaque assay (Jerne & Nordin, 1963).

Enumeration of PFC numbers was performed by adding 100 µl recovered kidney cells ($1–4 \times 10^6$ cells/slide) to an agarose mixture (i.e., 1% agarose, L-15 medium, and 10% sRBC) maintained in a 45°C waterbath. Tubes containing the agar were vortexed briefly, the agar was spread evenly over clean microscope slides, and the slides were then incubated for 4 h in a humidified 30°C chamber. Following incubation, slides were flooded (0.9 ml) with guinea pig sera (as a complement source) diluted 1:30 in PBS, and then incubated for an additional 4 h. The number of plaques on each slide was then counted using a Leitz binocular dissecting microscope. True plaques were differentiated from air bubbles by the presence of centrally located B cells and their faded, rather than defined, margins. Numbers of

plaques were scored relative to the total number of kidney lymphocytes plated per slide.

Host Resistance Infectivity Studies

Pilot studies were performed prior to the actual experiments to determine that concentration of *Y. ruckeri* needed to produce 50% lethality in adult medaka; the LD50 value was calculated by probit analysis using SAS PROC probit computer software. To assure pathogenicity, stability, and titer of the frozen bacterial stock, 2 groups of 10 medaka each were inoculated with an LD50 dose of previously frozen bacteria 3 d prior to the actual experiments.

For subsequent experiments, fish exposed to either clean well water or to waterborne malathion (i.e., 10 fish/aquaria; 40 fish/treatment) for either 7, 14, or 21 d were anesthetized lightly by a quick immersion into MS-222 (3-aminobenzoic acid ethyl ester; Sigma Chemical) and then injected ip (20 µl) with 1.7×10^7 colony-forming units (CFU) of viable bacteria. Seventy-two hours post-injection, bacteria were isolated from the kidneys of sentinel medaka to ensure infection of the host. Fish injected with *Yersinia* were monitored 3 times daily over a period of 4 d; all moribund/dead fish were removed at each inspection and the remaining numbers of fish were counted to determine percent cumulative mortality. To assure that water quality values remained within acceptable limits and that high loads of waterborne bacteria from excreta were minimized, one-half the volume of holding water was removed daily and replaced with fresh well water.

Statistical Analysis

The experimental design for both sets of experiments was a one-way treatment structure in a randomized incomplete block design structure with treatments at three levels (untreated control, "low" and "high" malathion doses) randomly assigned to four replicate tanks at each level. In all cases, mean values expressed were the result of two to four experiments. Significance was accepted at $p \leq .05$.

Ex Vivo Studies Probit analysis was used to estimate the LC50 malathion concentration in studies examining effects upon immune function; regression analyses was used to estimate the means and their associated 95% confidence limits for determination of the NOEL. SAS PROC probit and SAS PROC GLM computer software were used for all of this statistical analysis. Two-way analysis of variance (ANOVA) and the Tukey multiple comparison test were used to determine the effects of exposure concentration and duration between malathion-exposed and control groups. SPSS computer software was used to perform all of the analysis of variance calculations.

In Vivo Infectivity Studies Probit analyses was used to estimate the LD50 value for *Y. ruckeri* infection in normal fish; SAS PROC probit computer software was used to perform this analysis. The effects of toxicant concentration and exposure duration on medaka susceptibility to bacterial

infection were analyzed using two-way ANOVA and the Tukey multiple comparison test when appropriate; ANOVA was performed using SPSS computer software. Percent mortality was arcsine transformed for analysis and back-transformed to the arithmetic scale for presentation.

RESULTS

Ex Vivo Studies

In the first series of experiments, signs of intoxication were observed in some fish exposed to the highest tested malathion concentration (i.e., 0.8 mg/L); in some cases, signs appeared as early as 8 d after initiating exposure (data not shown). Intoxicated fish exhibited abnormal behavior (i.e., erratic swimming, inability to evade obstacles), neurological disturbances, and reduced food consumption. No overt signs of intoxication were observed in medaka exposed to the lower tested malathion concentration (i.e., 0.2 mg/L).

Exposure of fish to either uncontaminated well water or to water containing 0.2 (±0.05) mg malathion/L had no significant effect upon host survival. In contrast, exposure to a four-fold higher concentration (i.e., 0.8 ± 0.18) mg/L reduced fish survival by ~20% (compared to control) over 96 h (data not shown); hematocrit and total recoverable kidney cell numbers and viability were slightly reduced in these same fish, albeit not significantly (Table 1).

Effects of waterborne malathion exposure upon kidney phagocyte-generated reactive oxygen intermediates (ROI) were also examined in this study. Although the results failed to reach statistical significance, production of extra- (Figure 1A) and intracellular $O_2^{\bullet -}$ (Figure 1B), as well as H_2O_2 (Figure 2), by kidney phagocytes recovered from medaka exposed to 0.8 mg malathion/L was decreased compared to control values; extra- and intracellular $O_2^{\bullet -}$ production decreased by ~16%, while production

TABLE 1. Effects of Waterborne Exposure to Malathion at 0.8 mg/L (High) and 0.2 mg/L (Low) on the General Health Indices of Japanese Medaka

Parameter	High concentration	Low concentration	Untreated control
Length (mm)	28.2 ± 2	28.7 ± 2	28.2 ± 2
Weight (mg)	437.2 ± 92	441.4 ± 106	432.6 ± 109
Cell viability (%)	86.7 ± 8	91.1 ± 3	94.1 ± 1
Cell yield/fish ($\times 10^5$ cells)[a]	7.3 ± 2	8.9 ± 1	8.6 ± 1
Hematocrit (%)	49.8 ± 7	52.4 ± 7	51.8 ± 6
Leukocrit (%)	0.3 ± 0.3	0.3 ± 0.2	0.3 ± 0.3

Note. Values represent mean of four experiments ± SD.

[a]Data represent average cell yield per fish (e.g., number of cells for 10 fish = $7.2 \times 10^6/10 = 7.26 \times 10^5$ cells/fish). Because of their small size, an average value was calculated.

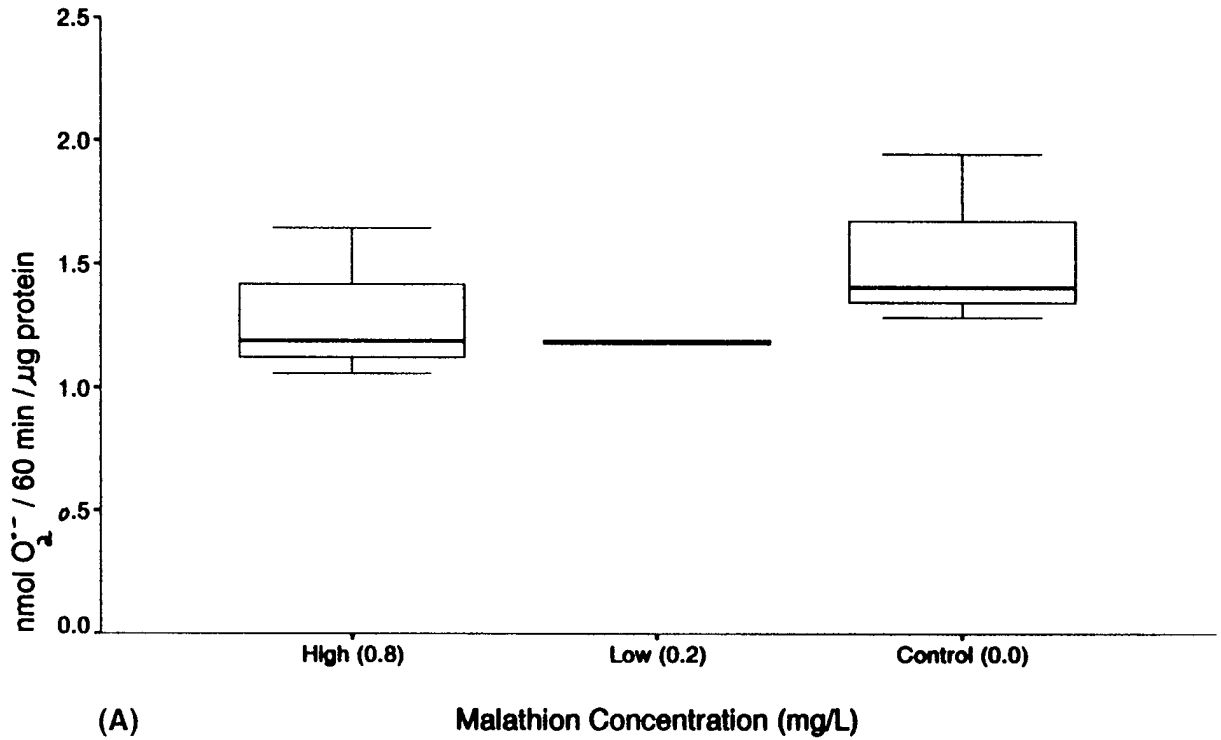

FIGURE 1. Production of (A) extracellular superoxide ($O_2^{\bullet-}$) by kidney phagocytes recovered from medaka exposed to either uncontaminated well water or to waterborne malathion at 0.2 (low) or 0.8 (high) mg/L is shown from these box plots. Extracellular and intracellular $O_2^{\bullet-}$ production was measured using ferricytochrome *c* and nitroblue tetrazolium (NBT) reduction, respectively. Values were calculated as nanomoles per microgram protein. No significant differences in either extracellular (*F* ratio = 1.5; *F* probability = 0.3) or intracellular $O_2^{\bullet-}$ production (*F* ratio = 0.2; *F* probability = 0.8) was observed between malathion-exposed and control groups. Except for the lowest malathion concentration, mean values (———) of extracellular $O_2^{\bullet-}$ were determined from 3 experiments (n = 3); mean value for the 0.2-mg/L exposure group was determined from 2 experiments. Values for intracellular $O_2^{\bullet-}$ represent the mean (———) of two experiments (n = 2) for the control and highest malathion group and one experiment for the 0.2 mg/L exposure group. Box around the mean (▭) represents the spread/variability of the observations. The upper and lower error bars represent the largest and smallest observed value, respectively, that was not an outlier.

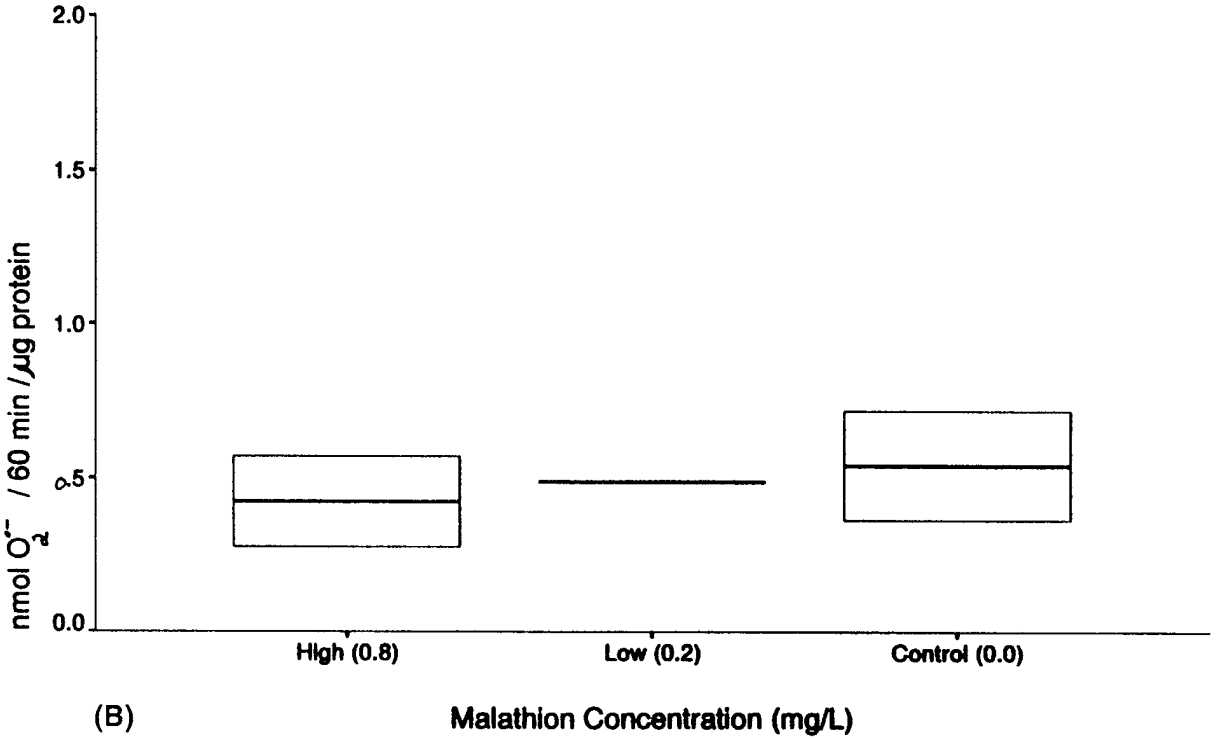

FIGURE 1. (*Continued*) Production of (B) intracellular superoxide ($O_2^{\bullet-}$) by kidney phagocytes recovered from medaka exposed to either uncontaminated well water or to waterborne malathion at 0.2 (low) or 0.8 (high) mg/L is shown from these box plots. Extracellular and intracellular $O_2^{\bullet-}$ production was measured using ferricytochrome *c* and nitroblue tetrazolium (NBT) reduction, respectively. Values were calculated as nanomoles per microgram protein. No significant differences in either extracellular (*F* ratio = 1.5; *F* probability = 0.3) or intracellular $O_2^{\bullet-}$ production (*F* ratio = 0.2; *F* probability = 0.8) was observed between malathion-exposed and control groups. Except for the lowest malathion concentration, mean values (———) of extracellular $O_2^{\bullet}$ were determined from 3 experiments (n = 3); mean value for the 0.2-mg/L exposure group was determined from 2 experiments. Values for intracellular $O_2^{\bullet-}$ represent the mean (———) of two experiments (n = 2) for the control and highest malathion group and one experiment for the 0.2 mg/L exposure group. Box around the mean (▭) represents the spread/variability of the observations. The upper and lower error bars represent the largest and smallest observed value, respectively, that was not an outlier.

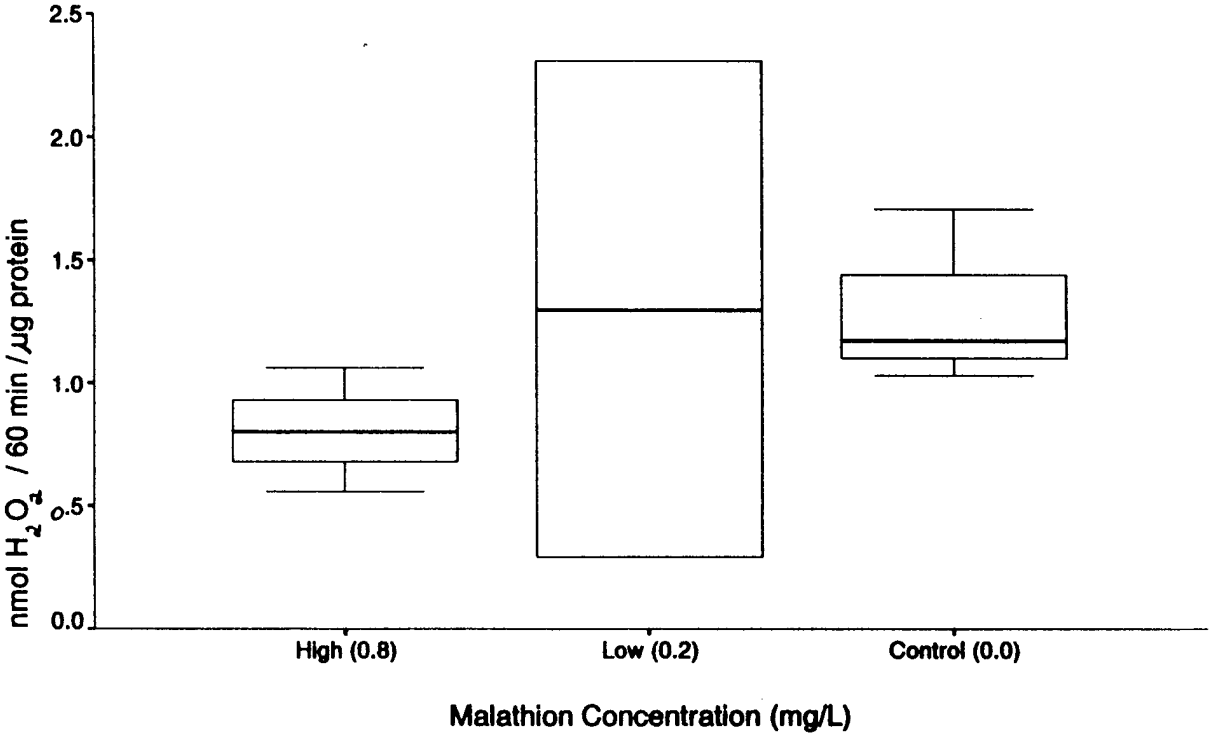

FIGURE 2. Effects of malathion exposure on hydrogen peroxide (H_2O_2) production by kidney phagocytes recovered from medaka exposed to 0.2 (low) or 0.8 (high) mg malathion/L. H_2O_2 formation was quantified by oxidation of phenol red and amounts were calculated as nanomoles H_2O_2 per microgram protein. No significant differences were observed between malathion-exposed and unexposed control groups (F ratio = 0.5; F probability = 0.6). Except for the lowest malathion concentration, mean values (———) of H_2O_2 were determined from three experiments (n = 3); mean value (———) for the 0.2-mg/L exposure group was determined from two experiments. Box around the mean (▭) represents the spread/variability of the observations. The upper and lower error bars represent the largest and smallest observed value, respectively, that was not an outlier.

of H_2O_2 decreased by ~32% compared to control values. Effects of malathion upon cell-mediated immunity were determined by examining pronephric T-cell proliferation in response to concanavalin A. Proliferative responses (measured in absorbance) in both malathion-exposed treatment groups were similar to that measured in the unexposed control fish (i.e., optical density = 0.15).

While exposure to malathion produced little or no effect upon non-specific and cell-mediated immune functions, B-lymphocyte activity was significantly reduced in malathion-exposed fish. Antibody PFCs recovered from the kidneys of malathion-exposed fish produced significantly fewer plaques in response to in vivo injection with sRBC than those produced by B-cells from unexposed control fish (Figure 3); the numbers of plaques decreased in a dose-response manner from 27 in the control group to 15 and 5 plaques/4 × 10^6 kidney cells in fish exposed to 0.2 and 0.8 mg malathion/L, respectively.

Host Resistance Studies

The mean 96-h mortality incidence for fish maintained in clean water for 7, 14, and 21 d and then infected with *Y. ruckeri* was 48, 41, and 38%, respectively. While no malathion-induced changes in host resistance against *Yersinia* were observed in fish exposed to malathion for 7 d (as compared to the unexposed infected control fish), longer malathion exposures significantly increased host susceptibility to infection (Figure 4); exposure of fish to malathion for either 14 or 21 d increased bacterial-induced mortality in a dose-dependent manner. Mortality incidence in medaka exposed to malathion for 14 d increased from 41% in the unexposed infected control fish to 58 and 78% in fish exposed to 0.1 and 0.3 mg malathion/L, respectively; in fish exposed to malathion for 21 d, bacterial-induced mortality increased from 38% (measured in the unexposed infected control fish) to 57 and 72%, respectively. Trauma-induced mortality related to injection was <5% for all exposure groups.

Statistical analysis evaluating the effects of malathion exposure on mortality incidence indicated that there was a significant concentration effect for both the 14 d (F ratio = 11.06; $p < .01$) and 21 d (F ratio = 13.03; $p < .005$) exposure groups at both malathion doses compared to control; no significant differences between 14- and 21-d treatment groups were observed.

DISCUSSION

The use of non-rodent species for immunotoxicologic evaluation of chemicals has gained increased attention (Zelikoff, 1994b; Luebke et al., 1997; Karol, 1998). While phylogenetically distant from humans, fish share a number of structural, functional, and biochemical characteristics important in the humoral, cell-mediated, and non-specific aspects of the

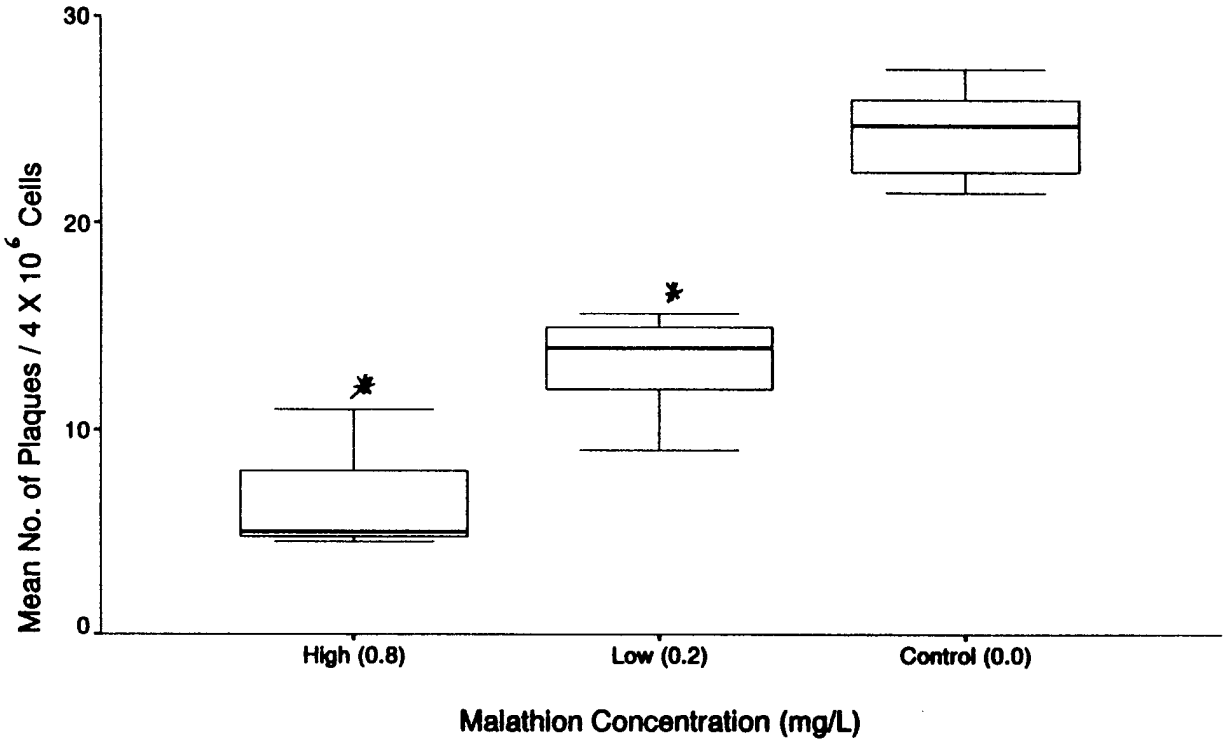

FIGURE 3. Effects of waterborne exposure to malathion on antibody plaque-forming cell (PFC) numbers in the kidney of control and malathion-exposed fish. A modified Jerne plaque assay was used to evaluate PFC response. Exposure to malathion produced a dose-dependent decrease in PFC numbers compared to those enumerated in the unexposed control fish. Asterisk indicates significant differences between both malathion exposure groups and control fish (F ratio = 41.4; F probability < .001). Values represent the mean of three to six experiments per exposure group. Box around the mean (⊟) represents the spread/variability of the observations. The upper and lower error bars represent the largest and smallest observed value, respectively, that was not an outlier.

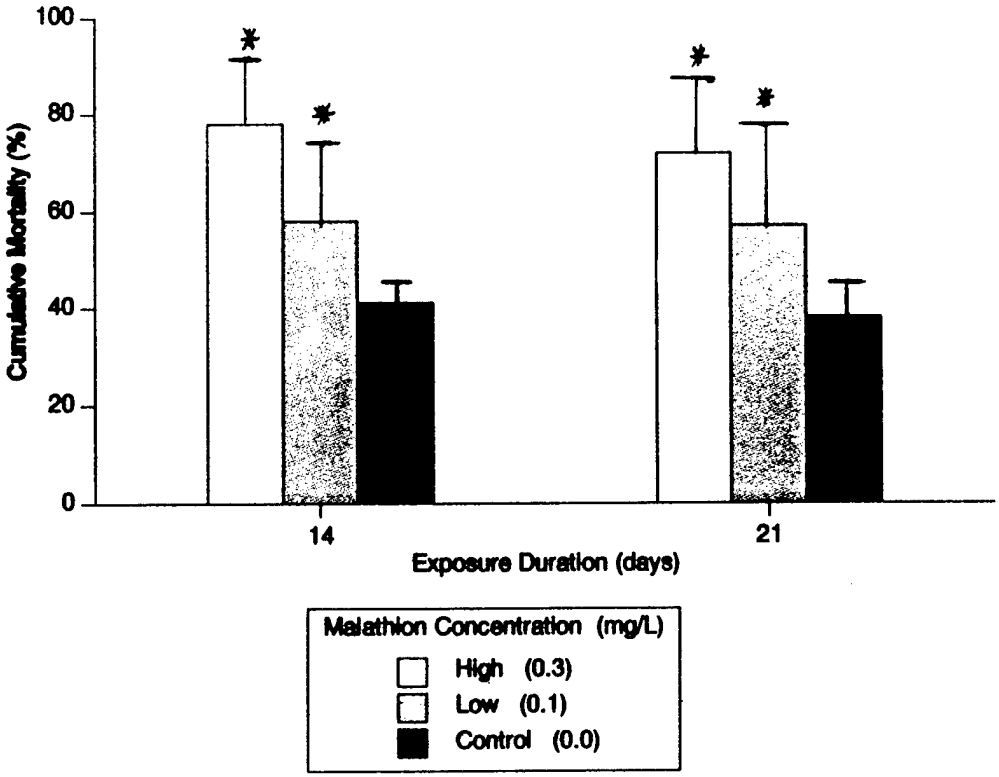

FIGURE 4. Effect of waterborne malathion exposure on host resistance against infection with *Yersinia ruckeri*. Exposure of medaka for either 14 or 21 d to 0.1 (low) and 0.3 (high) mg malathion/L increased malathion-induced mortality in a dose-dependent manner in exposed fish. Mortality incidence in the malathion-exposed groups was significantly different from that measured in the unexposed control group. While no differences were observed between the 14- and 21-d exposure groups, asterisk indicates significant differences between malathion-exposed and control groups exposed for 14 d (F ratio = 11.06, $p < .008$) and 21 d (F ratio = 13.03; $p < .005$).

immune response (reviewed in Zelikoff, 1994b). For example, fish can exhibit inflammation and hypersensitivity responses, synthesize specific antibody, phagocytize and intracellularly kill invading pathogens, respond to mitogen stimulation with the same agents used to stimulate mammalian lymphocytes, exhibit a mixed leukocyte response, process and present antigen, and release biologic mediators such as cytokines, interleukins, and arachidonic acid metabolites (reviewed in Zelikoff, 1994b, 1998). Moreover, fish offer a number of advantages over the current immunotoxicological mammalian models (i.e., mouse and rat): (1) larger species (i.e., trout, catfish), which can easily be maintained in the laboratory and provide large numbers of immune cells for study; (2) fish are amenable to laboratory and field studies and thus can be exposed to toxicants in well-defined laboratory situations and under more "natural conditions"; (3) fish are less costly to purchase and maintain than their mammalian counterparts; and (4) results

obtained from studies using fish can also provide evolutionary reference points for other vertebrate studies.

This study was designed to investigate the utility of NTP-validated mammalian immune assays for demonstrating malathion-induced immunotoxicity in a laboratory fish model. Results from these studies will help to define a specific panel of immune assays that could potentially be used to assess toxicological hazards/potential health risks associated with pesticide-contaminated ground/surface water sources. The most significant findings that emerged from these studies demonstrated that while subchronic exposure of medaka to sublethal concentrations of malathion had little to no effect upon non-specific or cell-mediated immune functions, humoral-mediated immunity and overall host resistance against an infectious bacterial fish pathogen were dramatically reduced.

Malathion-induced alterations in humoral-mediated immunity were determined by examining effects upon the pronephric antibody PFC response. This assay, one of the most sensitive for demonstrating chemical-induced immune cell perturbation, has been shown in rodents to have the greatest predictive power of immunotoxicity, second only to enumeration of lymphocyte cell surface markers (Luster et al., 1992, 1994) .

In these studies, subchronic waterborne exposure of medaka to malathion altered B-cell activity and decreased the host's ability to synthesize/-secrete T-dependent antigen-specific antibodies. The ability of malathion to alter humoral-mediated immune functions has been observed previously in other fish species, as well as in mammals. For example, Plumb and Areechon (1990a) demonstrated that agglutination antibody titers against the infectious bacteria *Edwardsiella ictaluri* were depressed (compared to controls) in channel catfish following subchronic exposure to waterborne malathion at concentrations similar to—and above—those employed in this study (i.e., 0.5 and 1.7 mg/L vs. 0.2 and 0.8 mg/L, respectively). Moreover, administration of 5 to 10 mg malathion/kg/d for 5 to 6 wk significantly decreased serum antibody titers generated in rodents in response to *Salmonella typhi* vaccination (Desi et al., 1978). Results from the aforementioned mammalian and piscine studies demonstrate malathion's ability to suppress humoral immune defense mechanisms important for controlling infection by bacterial pathogens. In other mammalian studies, Casale et al. (1983) demonstrated that a single cholinergic dose of malathion (720 mg/kg) given to mice per os 2 d after sRBC injection suppressed the primary immunoglobulin M (IgM) response; following administration of multiple (apparently) non-cholinergic malathion doses, no effects were observed upon the secondary IgG response. The authors of this cited study noted that immune suppression only occurred following administration of a malathion dosage that produced cholinergic effects, and that prolonged (3–5 h) cholinergic poisoning produced by the sustained release of the cholinomimetic agent arecoline reduced the number of IgM PFC by 50%. Based upon these findings, it was concluded that immuno-

suppression associated with excessive cholinergic stimulation resulted either from the direct action of acetylcholine on the immune system or indirectly as a result of glucocorticoid release in response to toxic chemical stress associated with cholinergic poisoning.

Given that malathion appears to produce neurotoxicity in fish via mechanism(s) similar to those described in mammals (Shao-nan & De-fang, 1996), that the same malathion metabolite shown to inhibit mammalian AChE is also the most active in teleost systems (i.e., malaxon), and that neurological sequelae suggestive of malathion intoxication were observed in this study in medaka exposed to the highest concentration tested (i.e., 0.8 mg malathion/L), the same mechanisms underlying malathion-induced immunosuppression in mammals might also be operative in fish.

Because the PFC response requires a concerted effort from innate and adaptive immunity, determining the exact mechanism(s) by which malathion may have acted to suppress PFC numbers in medaka is difficult and requires further study. However, based upon information gained from mammalian studies (Holsapple, 1995), suppression likely occurred via a direct effect of malathion on the immunocompetent organs/cells themselves and/or through a malathion-induced change in host neuroendocrine status.

Results from the present studies demonstrate that subchronic exposure of adult medaka to non-lethal concentrations of malathion enhanced host susceptibility to infection with the opportunistic gram-negative fish pathogen *Y. ruckeri*. Although no other information is currently available regarding the effects of malathion on mammalian or teleost host resistance, similar immunosuppressive effects have been observed in mammalian studies following exposure to a more acutely toxic organophosphate pesticide, parathion. In such cases, exposure to parathion increased host susceptibility of mice to cytomegalovirus and increased the virulence of the gram-negative bacteria *Salmonella typhimurium* in rabbits (Fan, 1981; Raise, 1984; Fan et al., 1984). Although malathion and parathion appear to produce toxicity via similar modes of action (i.e., AChE inhibition), extreme care must be taken in attempting to extrapolate results from one species to another due to differences in such factors as pesticide pharmacokinetics and metabolism.

Discerning the exact mechanism(s) by which exposure to malathion may have acted to reduce host resistance in medaka is difficult, particularly since immunological responses associated with *Y. ruckeri* infection in fish have not been clearly elucidated. However, given the dramatic suppressive effects observed in this study upon the PFC response, it seems probable that compromised host resistance may have occurred, at least in part, via malathion-induced effects upon some aspect(s) of humoral immunity. Moreover, in light of the fact that ingestion of *Y. ruckeri* by fish phagocytes depends upon antibody production for opsonization of the pathogen (Griffin, 1983), any chemical that could act to alter this particular function could, potentially, reduce host resistance against *Yersinia* infection. What-

ever the underlying mechanism(s) may be for the cited effects, the ability of malathion to reduce host resistance against this infectious bacterial agent has far-reaching implications that could result, ultimately, in an increased incidence of morbidity/mortality in malathion-exposed feral fish populations.

Results from this study have clearly demonstrated the utility of mammalian immune assays to be used successfully in fish to demonstrate potential toxicological hazards associated with pesticides that may pollute aquatic environments. Of particular interest is the fact that the same immune assays shown to be most sensitive in mammals for demonstrating chemical-induced immunotoxicity (i.e., PFC response and host resistance) also appear to be the most indicative of the immunosuppressive effects of a test chemical like malathion in medaka.

Studies such as these that employ fish to assess chemical-induced immunotoxicity fit well into the newly emerging trends for immunotoxicity testing, which include more in vitro tests, greater use of computational methods, and the development and validation of non-mammalian alternative species (Karol, 1998). Moreover, the validity/accuracy of cross-species extrapolation studies is improved by the ability to assess chemical-induced immunotoxicity in different animal models using the same assays. From an ecotoxicological perspective, findings from this study add substantially to the limited knowledge regarding the effects of toxic substances on fish health in general, and on the immune system, in particular. While more investigations are clearly needed before an accurate assessment of risk to inhabiting aquatic species can be made, the ability to evaluate fish health from an immunological standpoint may be invaluable to many countries whose populations have become increasingly more dependent upon finfish produced by aquacultural methods. The stress upon fish subjected to high loading densities under grow-out conditions (Peters et al., 1988) and the proximity of aquacultural sites to agricultural areas where pesticide runoff may occur necessitate the presence of an immunosurveillance tool to ensure the control and prevention of disease in exposed fish populations.

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